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Relationship of fowl lymphomatosis to Rous sarcoma

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NOTE TO USERS

RELATIONSHIP OF FOWL LYMPHOMATOSIS TO ROUS SARCOMA

by

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I. INTRODUCTION

Fowl lymphomatosis is a neoplastic disease-complex of major economic importance to the poultry industry. Within this disease-complex are usually grouped the various pathologic manifestations which include neural, ocular, visceral and osteopetrotic lymphomatosis. Microscopically all these forms are generally characterized by the presence of the undifferentiated neoplastic lymphocyte.

Rather definite evidence incriminates the carrier hen which may transmit the infection through the egg to the chick. Consequently efforts are being directed toward the development of a suitable means for detecting such apparently normal lymphomatosis-carrier hens.

The rather long and variable incubation period which seemingly characterizes fowl lymphomatosis and the numerous difficulties associated with obtaining consistently susceptible lymphomatosis-free stock for inoculation tend to limit progress in the study of this disease. During the investigation of avian neoplastic diseases, a number of workers have observed that neutralizing antibodies for the Rous sarcoma agent were present in the sera of certain apparently normal chickens. Some workers believe that latent sarcomatous agents are widespread in avian populations, although no neoplastic tissue changes are necessarily observed. Certain avian families and lines characterized by a high incidence of fowl lymphomatosis also exhibit definite neutralizing antibodies for the Rous sarcoma agent in many of their sera.

Should there be an antigenic relationship between the Rous sarcoma and the lymphomatosis-complex, the task of detecting lymphomatosis-carrier

hens through the use of the Rous virus neutralization test might be greatly facilitated. The Rous sarcoma agent usually produces a large tumor and death after a relatively short incubation period of twelve to twenty-one days. A minimal infective dose (MID) based on a 50 per cent lethal endpoint (LD_{50}) can be consistently demonstrated with cell-free extracts. Such an endpoint favors the detection of neutralizing antibodies in the serum.

The objective of the present study was directed toward a clarification of the antigenic relationship between fowl lymphomatosis and the Rous sarcoma, based on the occurrence of Rous neutralizing antibodies, the tissue pathology and the flock history. The incidence of Rous neutralizing antibodies was also determined in certain cases of experimental visceral lymphomatosis and in birds inoculated with lymphoid tumor preparations obtained from different flocks.

II. REVIEW OF LITERATURE

Carrel (1925) was the first to report the presence of neutralizing properties for the Rous agent in the blood of "normal" chickens. While studying the inhibiting effects of avian serum on the spontaneous inactivation of the Rous agent, he observed that sera from young chickens favored the development of larger tumors than sera from older chickens.

Fischer (1927) believed that 40 per cent of the normal chickens have a "virus-antagonistic principle" in their sera. This conclusion was based upon his studies concerning the neoplastic activity of cell-free material from Rous sarcoma tissue cultures.

Out of thirty-eight apparently normal chickens, Andrewes (1931) described two with antibodies against the Rous agent. He found no neutralizing antibodies in the sera from a hen with a non-transmissible round celled sarcoma on the back or from two hens with non-propagable angiosarcomas on the back of the neck.

The protective action of normal chicken serum was considered to be nonspecific by Sittenfield et al. (1931). These workers used 2 to 4 ml. of serum against one or two infective tumor doses. Likewise Des Ligneris (1934) suggested that the protective action of avian sera existed nonspecifically in normal sera. However the large quantities of inoculum used in Des Ligneris' study and the large volume of serum and small quantity of virus used by Sittenfield et al., would not favor the detection of moderate to low levels of neutralizing antibodies.

In (1933) Andrewes referred to a few "normal" fowl sera with Rous

neutralizing antibodies (RNA); such antibodies he later (1936) considered as developing normally in many fowl as they matured.

McMaster et al. (1934) reported that RNA were not demonstrated in the sera of day-old chicks, although often observed in sera from normal adult Plymouth Rock fowls and occasionally in pullets from the same flock.

Amies (1937) in studying the action of avian sera on the Rous sarcoma agent observed that the agglutinating antibody level paralleled the neutralizing antibody level. Forty-six normal chickens, eight to twelve weeks of age, gave negative results with the Rous agglutination test, while eight out of twenty adult Brown Leghorns were positive. It is interesting to note that none of these birds had come into contact with tumor bearing birds according to the history.

Then in (1939) Andrewes reported the occurrence of RNA in the sera of young "normal" chicks recently hatched from eggs laid by hens with a "natural" neutralizing antibody level in their sera. He believed that these antibodies were transferred through the egg yolk and persisted in the chick sera for a few weeks. He considered the RNA levels in apparently normal fowls to be of low potency following his study of forty-one Rous positive chickens hatched from hens and roosters whose sera contained RNA and from stock whose sera did not contain such neutralizing antibodies. Andrewes reported that only an occasional bird developed a relatively high neutralizing titer and that one of four controls developed Rous neutralizing antibodies at three months of age with an increase in titer at five months. Such observations led Andrewes to believe that viruses related to the Rous agent are widely

distributed and harmless.

Duran-Reynals (1940) tested numerous sera from young chicks and adult chickens for RNA. He found that twenty-five of twenty-seven sera from adult chickens were capable of neutralizing this agent, while sera from newly hatched chicks showed only occasional neutralization. He believed that the RNA level of a chicken's serum was directly proportional to the degree of resistance exhibited to the Rous agent. Such resistance was not attributed to serum antibodies by Carr (1943) who developed a nonsusceptible inbred line of Brown Leghorns, in which Rous sarcoma tumors regressed after twenty days.

The earliest report correlating tissue pathology with the RNA level in the sera was that of Andrewes (1931), who found no demonstrable RNA in the sera of a hen with a large spontaneous round celled sarcoma. He also found no RNA in the sera of two hens with small angiosarcomas on the back of the neck.

Cottral and Winton (1948) referred to Duran-Reynal's observation of RNA in the sera of chickens with fowl lymphomatosis. These chickens were from certain lines maintained at the Regional Poultry Research Laboratory, East Lansing, Michigan. According to Winton (1950) RNA have been observed in avian sera from Line 15, characterized by a 54 per cent incidence of fowl lymphomatosis in two years, while sera from isolated stock of the same line failed to have this neutralizing power. Yet when extracts of lymphoid tumors were inoculated into these isolated birds of Line 15, neutralizing antibodies for the Rous virus developed.

Duran-Reynals (1950) has observed a many-fold increase of RNA over a period of five months in chickens maintained in an animal room with

chickens and ducks supporting lymphoid and connective tissue tumors.

Andrewes (1936) observed that RNA were produced in pheasants by the inoculation of a tar-induced fowl sarcoma. Since the inoculation of fowl embryonic tissues did not stimulate RNA, Andrewes believed that these antibodies were of an antiviral nature, stimulated by a latent sarcoma virus present in the chemically-induced fowl sarcoma.

Foulds (1937) found that non-infective extracts of a tumor induced by 1:2:5:6: dibenzanthracene produced RNA when inoculated into rabbits.

Two grades of collodion membranes based on different pore sizes of 0.8 μ and 0.14 μ respectively were used by Foulds and Dmochowski (1939) in obtaining filtrates for the antigenic study of a non-filterable dibenzanthracene fowl sarcoma (RFD2) and the filterable Rous sarcoma No. 1. Complement fixation showed that RFD2 sarcoma and the Rous sarcoma contained two antigens not present in normal tissues, one antigen appeared comparable in size to the Rous agent, while the other appeared smaller than the Rous virus. Berkefeld filtrates of RFD2 were capable of stimulating RNA in the rabbit.

Dmochowski and Knox (1939) reported that serum from a rabbit immunized with 0.8 μ collodion membrane filtrates of a non-filterable dibenzanthracene-induced sarcoma RFD2 gave complement fixation with active filtrates of the Rous sarcoma, MH_2 tumor (filterable fibrosarcoma) and of fowl and duck grown Fujinami tumor. It is interesting to note that some of the RFD2 collodion filtrates failed to give a positive complement fixation test, which might be expected if the tumor host was not harboring a latent sarcomatous agent.

Gottschalk (1943) studied a methylcholanthrene-induced tumor (S16)

which stimulated the production of neutralizing antibodies for the filterable fowl sarcoma (S13) of Stubbs and Furth. These neutralizing antibodies for S13 were observed in the sera of chickens with S16 regressing tumors and in rabbits inoculated with high speed sediments of S16. Gottschalk offered four possible explanations: (1) S16 cells were accidentally associated with a filterable agent different from S13 but containing a related antigen; (2) contamination of S16 with the erythroleukosis Agent I, as the original bird was used for methylcholanthrene inoculation after failing to respond to inoculation of Agent I; (3) S16 was due to a filterable agent which has not been isolated; and (4) a specific antigen in S16 cells, which when isolated, was unable to initiate a malignant process.

Chemically-induced tumors have been demonstrated to act as havens for the Rous agent, Mellanby (1938). He also observed that no change occurred in the microscopic picture of the chemical tumor, although cell-free filtrates of the first and second generations of this tumor contained the Rous agent.

Carr (1942) suggested that the negative results of some workers studying the filterability of chemically-induced tumors may indicate that no latent sarcomatous agent was present. He also studied (1946) the dissemination of the Rous virus from a filterable to a non-filterable tumor in the same host. Carr found only about fifty minimal infective doses (MIDs) of Rous virus per gram of the non-filterable GRCH15 and MCaI tumors, although the Rous tumor itself contained more than 200,000 MIDs per gram. He was unable to demonstrate RNA in the sera of fowls inoculated with cells of non-filterable chemically-induced tumors GRCH15

and MCaI.

McIntosh (1933) reported that three of four tar-induced tumors were transmitted by means of cell-free Berkefeld filtrates. These transmitted tumors developed in sites other than that of inoculation, while leukemia was also present in some of his experimental chickens. McIntosh believed that the tar-stimulated neoplasm afforded young cells of mesoblastic origin, as a medium for invasion by sarcomatous and leukemic viruses, which he considered as being widely distributed in the avian world. In contrast, quite a number of investigators Murphy and Landsteiner (1925), Sturm and Murphy (1928), Peacock (1935), Mellanby (1938), Rothbard (1939) and Murphy and Sturm (1941) all failed to demonstrate filterability of chemically-induced tumors.

Apparently, latent tumor viruses may exist in the tissues of the chicken without exhibiting neoplastic changes, for McIntosh and Selbie (1939) have observed the appearance of only a sarcoma in the third passage of leukemic cells. They believed that two viruses were present and that a tumor agent might be carried over in a "leukemic condition" without producing a sarcomatous tumor.

Carr (1942) has reported the presence of Rous virus in a host for over a year before neoplastic changes occurred.

Through the inoculation of adult birds, Milford and Duran-Reynals (1943) have observed tumor activity in material from the sixth serial passage of the Rous sarcoma in chick egg embryos, although the embryos themselves exhibited no neoplastic tissue changes.

Greenwood and Peacock (1945) emphasized that the new host and the inoculum must be chosen with definite consideration for the history of

the flock involved, as some birds constitute fresh sources of sarcomatous agents.

Claude and Murphy (1933) have compiled pertinent data concerning twenty-eight transplantable tumors of the fowl, nineteen of which were proven filterable. Other filterable tumor strains of the fowl reported since this review of Claude and Murphy (1933) include Strain 13 of Stubbs and Furth (1935), Strains 11 and 15 of Furth (1936), Strain 12 by Furth (1936a), five sarcomas and fibromas by Duran-Reynals (1946), and the filterable lymphoid tumor agent reported by Burmester et al. (1946).

McIntosh (1933) suggested that a pleomorphic virus with affinity for any mesoblastic tissue might cause the formation of an endothelioma, a fibrosarcoma or leukemia.

Oberling and Guérin (1933) described an agent which they believed capable of producing leukosis, sarcoma and possibly a carcinoma.

Rothe Meyer and Engelbreth-Holm (1933) described an agent which produced a sarcoma and leukosis but not a carcinoma.

Troisier reported (1934) that the agent producing leukosis also produced a sarcoma and a carcinoma. The incidence of tumors in the controls was not cited.

Strain 13 of Stubbs and Furth (1935), which exhibited diffuse sarcomatosis of endothelial structures and an erythroleukosis phase, appeared rather consistent over the eighteen-month period of observation.

Olson (1941) described a lymphoid tumor, which later served as a source of the filterable agent isolated by Burmester et al. (1946). This agent produced a high incidence of visceral and osteopetrotic lymphomatosis within six months after inoculation.

Burmester and Denington (1947) reported variations in the transmission of tumor filtrates from spontaneous cases of visceral lymphomatosis. The possibility of a "masking" or "neutralizing" agent was considered.

Burmester (1947) suggested that a natural case of lymphomatosis may be associated with several tumor agents, which express themselves in a suitable host during subsequent transfers when conditions are favorable.

Tumor cells in an inoculum may be responsible for a neoplastic condition which differs from that observed when cell-free material is used. This was shown by Furth (1935) who described the occurrence of a lymphoid sarcoma when a cell-containing inoculum of Strain 2 was given intramuscularly, while cell-free material produced only lymphatic leukemia.

Pikovski et al. (1947) reported the appearance of a fibrosarcoma when cell-containing tumor material of the Engelbreth-Holm leukosis Strain 1 was inoculated intramuscularly, while cell-free material produced pure erythroleukosis.

III. MATERIALS AND METHODS

A. Rous Virus Neutralization Test

1. Preparation of the tumor suspension

A desiccate[1] of the Rous sarcoma tumor was suspended in saline and inoculated subcutaneously into several two-week-old White Leghorn chicks[2]. After twelve to fourteen days the chicks were sacrificed and neoplastic tissue from each of the inoculated chicks, free of blood and necrotic tissue, was collected in a covered sterile beaker partially immersed in water and ice. According to Duran-Reynals (1949) more consistent tumor activity was obtained from different tumor preparations through the harvest of a number of chicks of the same age after a uniform incubation period. Carr (1943) reported that between 10,000,000 and 100,000,000 infective units per gram of tumor tissue were present after fourteen to twenty-two days of incubation. The pooled tumor tissue was suspended in sufficient KCN-Ringer saline, pH 7.2-7.4, to give a 10 per cent dilution. The KCN-Ringer saline was prepared as follows:

NaCl	9.00 gm.
N/10 KCN	40.00 ml.
N/10 HCl	32.00 ml.
KCl	0.30 gm.
$CaCl_2$	0.25 gm.
$NaHCO_3$	0.20 gm.
Distilled water	1000.00 ml.

Pirie (1931) employed KCN-Ringer saline as a diluent for the Rous

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[1]Tumor desiccate was obtained from Dr. Duran Reynals, Yale School of Medicine, New Haven, Conn. through Dr. George Cottral of the Regional Poultry Research Laboratory, East Lansing, Michigan.

[2]Chicks were obtained from an inbred flock of White Leghorns maintained at the Veterinary Research Institute, Ames, Iowa.

agent. Dmochowski (1948) found KCN-saline quite suitable for suspension of the Rous virus in his studies.

Gye and Purdy (1930) have pointed out the protective value of HCN against loss of tumor activity due to oxidative changes.

Tumor tissue and diluent were homogenized in a cold Waring blendor[1] for 5 min. at a temperature of 4° C., then stored at 4° C. for at least four hours. Duran-Reynals (1940) has observed that the preparation and storage of tumor tissue at reduced temperatures favors maintenance of activity.

The homogenized tumor suspension was filtered through two layers of sterile gauze to obtain the filtrate which served as an inoculum to produce Rous tumors for harvest in twelve to fourteen days. After centrifugation[2] of the gauze filtrate for 30 min., 4° C., at 8-10,000 r.p.m. (Relative centrifugal force of approximately 5000 x gravity) to remove cells, the supernatant was used as a source of Rous virus for the neutralization test and for the determination of the minimal infective dose (MID) based on the LD_{50} according to the method of Reed and Muench (1938).

2. Technic of the Rous virus neutralization test

The cell-free Rous virus suspension was diluted with KCN-Ringer saline solution containing 0.04 per cent bovine albumen[3] to give a virus

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[1] Obtained from the A. H. Thomas Co., Philadelphia, Pa., No. 4281-E.

[2] Refrigerated Centrifuge, Model PR-1. International Equip. Co., Boston, Mass.

[3] Crystallized bovine plasma albumen. Armour Laboratories, Chicago, Ill.

dilution of 1:25,000. Bovine albumen was used as a protective for the virus, as Gye and Purdy (1930) found that serum of most animals would prevent the inactivation of Rous virus. Gottschalk (1946) found 2 per cent rabbit serum protective. Assays of tumor activity in Rous virus suspensions, diluted with bovine albumen, have been observed to give a more consistent and higher MID. The protection afforded the virus was more comparable to that offered by the avian serum proteins present in the virus neutralization tests.

The avian sera to be tested were first diluted 1:2 with 0.85 per cent saline or used without dilution. If neutralizing properties are present and the information is desired, higher dilutions than 1:2 may be carried out later in five-fold steps. Sera may be stored in an electric or dry-ice deep freeze until needed. Equal volumes of the 1:25,000 virus dilution and 1:2 serum dilution were mixed together to give a total volume of 2 ml. and final dilutions of 1:50,000 and 1:4 respectively. The serum-virus mixtures were placed in the refrigerator, 4° C., overnight or for at least four hours. This method was used by Duran-Reynals (1940) who found that Rous virus inactivation occurred at ordinary room temperatures. After incubation at 4° C., a 0.2 ml. volume of the serum-virus mixture was inoculated subcutaneously, over the pectoral muscles of one side, into each of five two-week-old White Leghorn chicks. Duran-Reynals (1949) inoculated four to six subcutaneous sites in each chick with the control virus and different serum-virus mixtures.

The number of minimal infective doses (MIDs) employed in this study varied from 3 to 140, depending on the activity of the particular tumor

harvest. The inoculum was placed subcutaneously in such a manner that no loss of the serum-virus mixture occurred through the site of needle entry. This was done by the direction of the needle point (24 gauge 1.5 in.) through the skin and partially into the pectoral muscles and then back up next to the skin some distance from the site of entry. During the two to three hour period required for chick inoculations, serum-virus mixtures were kept in an ice-water bath. The inoculated chicks were identified by wingbands and kept under observation thirty days for tumor development. When no neutralization occurred, many of the chicks developed extensive Rous tumors within eighteen to twenty-four days and died. All chicks surviving after thirty days were sacrificed and the extent of tumor development was evaluated. Duran-Reynals employed an incubation period of thirty-two days (1949).

3. Evaluation of Rous tumors

Tumors were classified on the following basis: (a) 4+ referred to an extensive tumor growth involving the pectoral muscles on the inoculated side, which distended the skin so that the chick walked in an unbalanced manner. The tumor growth usually contained a large amount of a hemorrhagic exudate with a subcutaneous edema present over the abdominal muscles and over the muscles of the uninoculated side. Chicks supporting this type of tumor growth always die. (b) 3+ referred to a type of tumor development much like that in the 4+ group, except that distension of the skin was moderate in extent. This type of tumor usually developed into a 4+ response. (c) 2+ referred to a much less pronounced tumor development, essentially characterized by a hemorrhagic border along the

periphery of the neoplastic tissue or a group of such tumors distributed over several square inches of the breast musculature. Usually this type of tumor developed into a 3+ or 4+ response. (d) 1+ referred to a tumor without a hemorrhagic border. These tumors may enlarge to an inch or so in diameter but usually regress without causing death. Many of these tumors are one-fourth to one-half inch in diameter and quite hard. (e) (-) No tumor development present.

Claude (1937) utilized an index of activity to evaluate the extent of tumor formation. This index depended on the size of the tumor and the number of takes. Dmochowski (1948) employed a similar procedure. Duran-Reynals (1940) and (1949) measured the area of tumor growths comparing controls with the inoculates. He considered that neutralization of the Rous virus occurred when the tumor size was equal to less than half the mean area of all the control tumors in the group.

4. Computation of the tumor index (TI)

The tumor response of the five inoculated chicks as a group was evaluated by the use of the TI in an attempt to determine if neutralization had occurred. If so, no tumor development was usually observed. However, certain sera with some neutralizing properties allowed only partial tumor development. The sum total of the quantitative measurements of tumor development in each of the five chicks was divided by the number of observations to obtain the TI. Values of 0.0-2.0 were considered as evidence that the serum being tested contained neutralizing antibodies. An occasional chick was observed to be unusually susceptible to the Rous virus, as was suggested by the appearance of an occasional tumor in the

1:31,250,000 dilution during the assay of tumor activity. Similarly some chicks appeared quite resistant.

5. Determination of the minimal infective dose (MID)

The MID as employed in this study referred to the greatest dilution of Rous virus capable of producing at least a 2+ type tumor in 50 per cent of the chicks inoculated within thirty days. The cell-free Rous virus suspension was diluted with KCN-Ringer saline containing 0.04 per cent bovine albumen, to give five-fold dilutions of 1:250,000, 1:1,250,000, 1:6,250,000 and 1:31,250,000. These dilutions were made up at the same time and treated in the same way as the serum neutralization tests and placed in the refrigerator overnight or for at least four hours. Two-week-old chicks were inoculated subcutaneously on one side over the pectoral muscles with a 0.2 ml. quantity of the Rous virus. Groups of five to seven chicks were inoculated with each of the above virus dilutions. After an observation period of thirty days, the surviving chicks were sacrificed and the extent of the tumor development was noted. Occasionally a tumor developed in one of the chicks inoculated with the 1:31,250,000 virus dilution while usually all of the chicks inoculated with the 1:250,000 dilution of virus developed tumors. The MID based on the LD_{50} endpoint varied with the different tumor preparations but usually fell within the dilution range of 1:1,250,000 to 1:6,250,000. This corresponded to Carr's report (1943) of between 10,000,000 and 100,000,000 infective units per gram of tumor tissue. Carr (1942) determined the MID by inoculating successive decimal dilutions of Rous virus into the breasts and legs of six-week-old susceptible chicks.

6. Determination of neutralizing doses (NDs) per ml. of serum

A neutralizing dose of serum was the amount of serum necessary to prevent one MID from developing more than a 1+ tumor. This value was based on the MID of the particular virus preparation employed and the highest dilution of serum exhibiting definite neutralization. In the following example a serum specimen exhibited neutralization at the 1:4 dilution and the MID was present in the 1:6,250,000 dilution group. The final dilution of Rous virus employed in this test was 1:50,000. The factor of one-half was used since the MID was based on a 0.2 ml. quantity of virus. Since 0.1 ml. of serum was used in the neutralization test, results were multiplied by 10 for a 1 ml. quantity. The factor of 4 referred to the dilution of serum used in this test. The NDs were calculated from the following:

$$\frac{6,250,000}{50,000} \times \frac{1}{2} \times 10 \times 4 = 2500 \text{ NDs per ml. of serum.}$$

B. Pathology

Any changes suggestive of disease were noted in all the chickens examined. At the time of autopsy, lesions were observed and representative tissues collected for microscopic study. Such tissues were first placed in a formalin fixative for twenty-four hours. This fixative consisted of 10 per cent formalin in physiological saline with calcium carbonate chips added as a buffer. The tissue blocks were then placed in 70 per cent alcohol for twenty-four hours and transferred to 95 per

cent alcohol. After twenty-four hours, the blocks were placed in absolute alcohol for eight hours. They were then transferred to chloroform and fifteen hours later placed in a chloroform-paraffin mixture (chloroform saturated with melted paraffin) for four hours at 56° C. Following this they were transferred to Altmann's mixture, in which they were embedded and cooled after four hours. Altmann's mixture consists of 850 gm. of paraffin, 50 gm. of stearin and 25 gm. of beeswax. These ingredients were melted together and the resulting liquid mixture kept at 56° C.

Tissue sections were cut at 5 μ thickness and routinely stained with Delafield's hematoxylin-ethyl-eosin stain and mounted in Permount[1].

The identification and classification of neoplastic changes were based on Feldman and Olson's discussion of avian neoplastic diseases (1948).

Super Panchro-Press Type B film was used for taking the gross pictures used in this study. Photomicrographs were taken of hematoxylin-eosin stained sections from the myxosarcoma used in producing the experimental cases of fowl lymphomatosis. Sections of tissues from chickens with fowl lymphomatosis in each of the first, second and third inoculation series were also photographed. Kodak M plates were used with Wratten B and G filters. Plates, film and paper were developed with Kodak Universal M-Q Developer. Pictures were printed on AD-type paper made

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[1] Obtained from Fisher Scientific Co., Pittsburgh, Pa. No. 12-568.

by Eastman Kodak.

C. The Occurrence of Rous Neutralizing Antibodies in the Sera from Different Types of Flocks

1. Flocks with a 20 to 50 per cent incidence of fowl lymphomatosis

Sera and tissues were usually collected from chickens submitted by flock owners and veterinarians. Some of these flock histories were made available by the attending veterinarian. Otherwise, the observations of the owners were utilized in an attempt to evaluate the extent of losses due to lymphomatosis.

Flock S[1]: Pullets hatched in February 1950 (New Hampshire-Leghorn cross) began to die in early September from visceral lymphomatosis. Removal of culls and dead birds had reduced the laying flock by 30 per cent. In October, thirty of these hens were purchased and transferred to the Veterinary Research Institute, Ames, Iowa, for observation and determination of Rous neutralizing antibodies (RNA).

Flock 454: Pullets hatched in March 1950 (White Leghorns) began to die in the latter part of August. The owner reported that the dead birds exhibited greatly enlarged livers. Three of the four birds submitted in September for examination exhibited typical visceral lymphomatosis. A loss of over 20 per cent was reported by late October 1950. Sera and tissues were collected.

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[1] Flock S was under the care of Dr. P. V. Neuzil, a veterinarian at Blairstown, Iowa, who posted many of the dead birds and made available the history of this flock.

Flock 494[1]: A loss of five hundred out of two thousand hens occurred since laying began. Many of these showed symptoms and changes suggestive of lymphomatosis. Bumblefoot was also observed at the time that three of the flock were submitted for postmortem examination.

Flock 526: White Leghorn pullets hatched in March 1950. Neural lymphomatosis was observed in July and August, while ocular cases were first noted in August. Losses from the visceral type began to appear in October and continued into the winter. After further culling for light birds and ocular lesions about 330 of the original 680 pullets were left. Four birds were submitted for examination.

2. Flocks with less than a 5 per cent incidence of lymphomatosis

Flock RG[2]: New Hampshire Reds with less than a 5 per cent loss due to the lymphomatosis complex. A number of culls and birds exhibiting the different forms of lymphomatosis were made available for this study.

Flock AI: Only an occasional case of fowl lymphomatosis was observed in this flock of New Hampshire Red hens.

Flock Q-1[3]: Two New Hampshire Red hens, eighteen months old, were obtained from a flock of 310. No losses due to lymphomatosis were noted.

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[1]Flock 494: These birds were examined by Dr. W. P. Switzer of the Iowa State Veterinary Diagnostic Laboratory, Ames, Iowa, who made available the tissues, sera and respective histories.

[2]Owned by Dr. Robert Getty, Dept. of Veterinary Anatomy, Iowa State College, Ames, Iowa, who maintained accurate records and posted all dead birds.

[3]This flock was under the care of Dr. P. V. Neuzil, a veterinarian at Blairstown, Iowa, who posted the dead birds and made available the respective histories.

Flock Q-2[1]: Three New Hampshire Red pullets, hatched in June 1950, were selected from a flock of 440. A White Leghorn rooster hatched and raised with these was also obtained. Sera were collected from these birds on various occasions, as well as, from the rest of the White Leghorn roosters[3] running with this flock on the range.

Flock G[2]: A flock of 110 pullets (Barred Rock and New Hampshire Red cross) hatched in March 1950. Ocular cases were observed in the flock by late September, while the visceral type had been observed in one bird. Sera were collected at various intervals from birds transferred to the Veterinary Research Institute.

Flock VRI: An inbred flock of 550 White Leghorn hens and roosters were maintained as a source of experimental birds and fertile eggs at the Veterinary Research Institute. Normal chickens as well as those exhibiting different types of lymphomatosis or symptoms of other diseases were bled to obtain sera for the Rous virus neutralization test. Hens and roosters from the hatches of 1948, 1949 and 1950, bled during the last eighteen months, were included in this study. Gross and microscopic examinations of birds, from this flock, indicated that there was less than a 4 per cent incidence of lymphomatosis during the last eighteen months.

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[1] This flock was under the care of Dr. P. V. Neuzil, a veterinarian at Blairstown, Iowa, who posted the dead birds and made available the respective histories.

[2] Ibid.

[3] Sera from the roosters were collected by Dr. P. V. Neuzil.

3. Two flocks hatched together, one RPL[1], from stock with a 41 per cent[2] incidence of lymphomatosis and the other VRI[3], from stock with less than a 4 per cent incidence of this disease

These chicks were raised together in brooders for five weeks and then placed in brooder houses with sun porches. About equal numbers of RPL and VRI chickens were housed in each brooder unit. Fifty per cent of the RPL population were inoculated with an attenuated Rous virus vaccine[4] in an attempt to alter the incidence of lymphomatosis. Unvaccinated isolated controls were placed in an individual brooder house at the time of vaccination. Losses of about 20 per cent due to coccidiosis and Newcastle disease were experienced. Most of the RPL birds were bled three times, while some were bled just once. Sera were first collected at eight weeks of age. The VRI birds were bled at seven months of age and again five months later.

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[1] Trapnested eggs, pedigree-hatched, from Line 9 of the Regional Poultry Research Laboratory, East Lansing, Michigan, were made available for hatching through Mr. Berley Winton, Director and Dr. N. F. Waters, Geneticist.

[2] This information was obtained from Mr. Berley Winton in a personal communication.

[3] Pedigree-hatched chicks from inbred White Leghorn families maintained at the Veterinary Research Institute.

[4] A methanol precipitated Rous virus vaccine treated with formalin so that 0.2 ml. given subcutaneously failed to produce tumors in two-week-old chicks. No neutralizing antibodies were demonstrated in sera from three-month-old test birds, bled three and eight weeks after vaccination.

D. The Occurrence of Rous Neutralizing Antibodies in Chickens Inoculated with Fowl Lymphomatosis Tumor Suspensions Obtained from Cases of Visceral Lymphomatosis[1]

Ten per cent suspensions of neoplastic livers were prepared in the Waring blendor, employing KCN-Ringer saline as a diluent. After two to three minutes of blending, the liver suspension was well homogenized without destroying many of the tumor cells. Chicks[2], three to fourteen days of age, were inoculated intraperitoneally with 0.5 ml. of this tumor suspension and raised in isolation. Sera were collected when these birds were four, eight and thirty-two weeks of age.

E. The Occurrence of Rous Neutralizing Antibodies in Experimental Cases of Fowl Lymphomatosis

1. First inoculation series

Visceral lymphomatosis appeared in some of the birds inoculated with a myxosarcoma[3] tumor suspension. This 10 per cent suspension was diluted in KCN-Ringer saline, comminuted in a Waring blendor and inoculated May 24, 1950, intramuscularly into five three-week-old White Leghorn chicks. Controls from the same hatch were raised separately. The inoculated

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[1] These cases of visceral lymphomatosis were obtained from birds in which no neutralizing antibodies for the Rous virus had been demonstrated.

[2] Chicks were pedigree-hatched from inbred White Leghorn families maintained at the Veterinary Research Institute.

[3] This tumor was present in an eight-week-old New Hampshire Red pullet, made available by Dr. W. P. Switzer of the Iowa State Veterinary Diagnostic Laboratory, Ames, Iowa.

chicks were raised in isolation and bled at various intervals. About five months later the affected liver from one of these birds with visceral lymphomatosis was utilized as an inoculum for the second series.

2. Second inoculation series

One of the first inoculation series birds, 654, was sacrificed October 10, 1950 and a tumor suspension prepared from the affected liver. Five three-day-old White Leghorn chicks were inoculated intraperitoneally with 0.5 ml. of the tumor suspension, while a similar number served as controls. The inoculated and the control chicks were raised together in a single pen and bled at various intervals. Tissues from one of these birds, H20, which developed visceral lymphomatosis December 29, 1950, served as an inoculum for the third series.

3. Third inoculation series

The birds inoculated with tumor material from H20 and the controls were bled at various intervals and observed for the appearance of visceral lymphomatosis. These chicks were one-week-old at the time of inoculation. Controls were raised in the same pen with the inoculated birds while another group of controls were raised in a separate building.

IV. RESULTS

Data obtained from the various flocks considered in this study are presented in the tables below. Evidence of neutralization is indicated by a tumor index of 0.0 to 2.0. Indices up to and including 2.8 are considered as questionable evidence of neutralization.

A. Flocks with a High Incidence of Lymphomatosis

Table 1. Results of the Rous Neutralization Tests Carried Out with Sera Collected at Various Intervals from Flock S.

Hen no.	Date bled	NR and SD[a]	MIDs[b]	NDs/ml. serum[c]	Tumor index	History and pathology
5	10-28-50	+ 1:4	35	1400	0.0	Appeared normal
	3-14-51	+ 1:2	140	2800	1.8	" "
6	10- 7-50	+ 1:4	130	5200	2.0	" "
	10-28-50	- 1:4	35		4.0	" "
	3-14-51	+ 1:2	140	2800	1.6	" "
7	10- 7-50	+ 1:4	130	5200	0.0	Died VL[d], 10-22-50
8	10- 7-50	+ 1:4	130	5200	0.0	Appeared normal
	10-28-50	+ 1:4	35	1400	0.0	" "
	3-14-51	- 1:4	140		3.6	" "

[a] Neutralization reaction and serum dilution.

[b] Minimal infective doses

[c] Neutralizing doses per ml.

[d] Visceral lymphomatosis.

Table 1. (Continued)

Hen no.	Date bled	NR and SD	MIDs	NDs/ml. serum	Tumor index	History and pathology
9	10- 7-50	- 1:4	130		4.0	Appeared normal
	10-28-50	- 1:4	35		4.0	" "
	3-14-51	- 1:2	140		4.0	" "
10	10- 7-50	+ 1:4	130	5200	0.0	" "
	10-28-50	+ 1:4	35	1400	0.0	" "
	3-14-51	+ 1:2	140	2800	0.0	" "
11	10- 7-50	- 1:4	130		4.0	" "
	10-28-50	- 1:4	35		4.0	" "
	3-14-51	- 1:2	140		4.0	" "
12	10- 7-50	+ 1:4	130	5200	0.0	Died VL, 10-15-50
13	10- 7-50	+ 1:4	130	5200	0.0	Appeared normal
	10-28-50	+ 1:4	35	1400	0.0	" "
	3-14-51	+ 1:2	140	2800	0.0	" "
14	10- 7-50	- 1:4	130		4.0	" "
	3-14-51	- 1:2	140		3.8	" "
15	10- 7-50	- 1:4	130		4.0	" "
	10-28-50	- 1:4	35		4.0	" "
	3-14-51	? 1:2	140		2.6	" "
16	10- 7-50	+ 1:4	130	5200	0.0	" "
	10-28-50	+ 1:4	35	1400	0.2	" "
	3-14-51	+ 1:2	140	2800	0.0	" "
17	10- 7-50	- 1:4	130		4.0	" "
	10-28-50	- 1:4	35		4.0	" "
	3-14-51	- 1:2	140		4.0	" "
18	10- 7-50	- 1:4	130		4.0	" "
	10-28-50	- 1:4	35		4.0	" "
	3-14-51	- 1:2	140		4.0	" "
20	10- 7-50	- 1:4	130		4.0	" "
	10-28-50	- 1:4	35		4.0	Died of hemorrhage, 1-12-50
21	10- 7-50	+ 1:4	130	5200	0.0	Lame
	10-28-50	+ 1:4	35	1400	0.0	Died. Peritonitis, 10-28-50

Table 1. (Continued)

Hen no.	Date bled	NR and SD	MIDs	NDs/ml. serum	Tumor index	History and pathology
22	10- 7-50	- 1:4	130		4.0	Appeared normal
	10-28-50	+ 1:4	35	1400	2.0	" "
	3-14-51	- 1:2	140		4.0	" "
23	10- 7-50	+ 1:4	130	5200	0.0	" "
	10-28-50	+ 1:4	35	1400	0.0	" "
	3-14-51	- 1:2	140		3.4	" "
24	10- 7-50	+ 1:4	130	5200	0.0	" "
	10-28-50	+ 1:4	35	1400	0.4	" "
	3-14-51	- 1:2	140		3.0	" "
25	10- 7-50	+ 1:4	130	5200	0.0	" "
	10-28-50	+ 1:4	35	1400	0.0	" "
	3-14-51	+ 1:2	140	2800	0.4	" "
59	10- 7-50	- 1:4	130		4.0	Died of hemorrhage, 10-8-50
60	10- 7-50	- 1:4	130		4.0	Early gray eye
	10-28-50	? 1:4	35		2.4	Died 11-2-50, VL & OL[a]
61	10- 7-50	- 1:4	130		4.0	Appeared normal
	10-28-50	- 1:4	35		4.0	Died of injuries, 3-14-51
62	10- 7-50	+ 1:4	130	5200	0.0	Appeared normal
	10-28-50	+ 1:4	35	1400	0.0	" "
	3-14-51	+ 1:2	140	2800	0.0	" "
63	10- 7-50	+ 1:4	130	5200	0.0	" "
	10-28-50	+ 1:4	35	1400	0.0	Died VL, 11-27-50
64	10- 7-50	- 1:4	130		4.0	Appeared normal
	10-28-50	- 1:4	35		4.0	Impacted oviduct
65	10- 7-50	+ 1:4	130	5200	0.0	Appeared normal
	10-28-50	+ 1:4	35	1400	0.0	" "
	3-14-51	+ 1:2	140	2800	0.0	" "
66	10- 7-50	+ 1:4	130	5200	0.0	" "
	10-28-50	+ 1:4	35	1400	0.0	" "
	3-14-51	+ 1:2	140	2800	0.0	" "
67	10- 7-50	- 1:4	130		4.0	" "
	10-28-50	+ 1:4	35	1400	1.6	" "
	3-14-51	- 1:2	140		3.2	" "
68	10- 7-50	+ 1:4	130	5200	0.0	" "
	10-28-50	+ 1:4	35	1400	0.0	" "
	3-14-51	+ 1:2	140	2800	0.0	" "

[a] Ocular lymphomatosis.

Table 2. Results of the Rous Neutralization Tests Carried Out with Sera Collected from Several Flocks.

Bird no.	Date bled	NR and SD[a]	MIDs[b]	NDs/ml serum[c]	Tumor index	History and pathology
454A						VL[d], dead
454B	9-16-50	+ 1:100	37	37,600	0.0	Hepatic hematomas
454C	9-16-50	+ 1:100	37	37,600	0.0	VL
454D						VL, dead
494A	10-23-50	+ 1:4	25	1020	0.0	VL
494B	10-23-50	+ 1:4	25	1020	2.0	VL
494C	10-23-50	+ 1:4	25	1020	0.0	Bumblefoot
526A	11-27-50	+ 1:4	25	1020	1.0	OL[e]
526B	11-27-50	- 1:4	25		4.0	Appeared normal
526C	11-27-50	- 1:4	25		4.0	OL & VL
526D	11-27-50	+ 1:4	25	1020	0.0	OL

[a] Neutralization reaction and serum dilution

[b] Minimal infective doses.

[c] Neutralizing doses per ml.

[d] Visceral lymphomatosis.

[e] Ocular lymphomatosis.

B. Flocks with a Low Incidence of Lymphomatosis

Table 3. Results of the Rous Neutralization Tests Carried Out with Sera Collected from the RG and AI flocks.

Bird no.	Date bled	NR and SD[a]	MIDs[b]	Tumor index	History and pathology
365	6- 6-50	- 1:4	72	4.0	Foot injured, 1949 hatch
	10- 7-50	- 1:4	28	4.0	
378	6-21-50	- 1:4	72	4.0	NL[c], five-weeks-old
379	6-21-50	- 1:4	72	4.0	" " " "
380	6-21-50	- 1:4	72	4.0	" " " "
495	10-24-50	- 1:4	28	4.0	OL[d], 1950 hatch
496	10-24-50	- 1:4	28	4.0	" " "
530	12- 5-50	- 1:4	3.3	4.0	NL
561	12- 5-50	- 1:4	6.0	4.0	NL
580	1- 3-51	- 1:4	6.0	4.0	OL
581	1- 3-51	- 1:4	62.5	4.0	VL[e]
587	1- 3-51	- 1:4	62.5	4.0	VL
588	1- 3-51	- 1:4	62.5	4.0	OL
591	1- 3-51	- 1:4	62.5	4.0	VL
592	1- 3-51	- 1:4	62.5	4.0	NL
598	1- 3-51	- 1:4	62.5	4.0	OL
622	2- 2-51	- 1:4	111.0	4.0	NL
540*	12- 1-50	? 1:4	3.3	2.4	Osteopetrotic lymphomatosis
583*	1- 8-51	- 1:4	12.5	4.0	" "

[a] Neutralization reaction and serum dilution.

[b] Minimal infective doses.

[c] Neural lymphomatosis.

[d] Ocular lymphomatosis.

[e] Visceral lymphomatosis.

*Birds from the AI flock.

Table 4. Results of the Rous Neutralization Tests Carried Out with Sera Collected from Several Flocks.

Bird no.	Date bled	NR and SD[a]	MIDs[b]	ND/ml? serum	Tumor index	History and pathology
Q39	10- 1-50	- 1:4	79.0		4.0	Depigmented eyes
&	10-28-50	- 1:4	25.0		4.0	
Q41						
Q42	10- 1-50	+ 1:100	79.0	79,000	0.0	Atypical eye color
	10-28-50	+ 1:4	25.0	1020	0.0	
	4-12-51	? 1:2	12.5		2.4	
5	1-17-51	+ 1:7	92.0	6440	1.6	Flock mate of Q42, male
17	1-17-51	+ 1:14	92.0	12,880	0.8	" " " "
25	1-17-51	+ 1:5	92.0	6440	1.6	" " " "
group*	1-17-51	- 1:4	92.0		4.0	Flock mates of Q42, male
Q44	10- 1-50	- 1:4	79.0		4.0	Atypical eye color
	10-28-50	+ 1:4	25.0	1020	1.0	
	12-26-50	+ 1:4	12.5	500	1.0	
	2- 9-51	- 1:4	28.0		4.0	
Q45	10- 1-50	- 1:4	79.0		4.0	Atypical eye color
	10-28-50	+ 1:4	25.0	1020	1.6	
	12-26-50	+ 1:4	12.5	500	1.0	
	2- 9-51	- 1:4	28.0		4.0	
Q46	10- 1-50	- 1:4	79.0		4.0	Atypical eye color
	10-28-50	+ 1:4	25.0	1020	1.4	
	12-20-50	- 1:4	12.5		4.0	Died VL[d], 12-20-50
G98	10- 1-50	- 1:4	130.0		4.0	Ocular lymphomatosis
	12-26-50	+ 1:4	12.5	500	0.0	
	2- 9-51	- 1:4	28.0		4.0	
G99	10- 1-50	- 1:4	130.0		4.0	Ocular lymphomatosis
	12-26-50	- 1:4	12.5		4.0	
	2-16-51	- 1:4	111.0		4.0	
G100	10- 1-50	- 1:4	130.0		4.0	Ocular lymphomatosis
	12-26-50	- 1:4	12.5		4.0	
	1-24-51	- 1:4	27.5		4.0	Died, 1-24-51

[a] Neutralization reaction and serum dilution.

[b] Minimal infective doses.

[c] Neutralizing doses per ml.

[d] Visceral lymphomatosis.

* Twenty-five sera tested individually.

Table 5. Results of the Rous Neutralization Tests Carried Out with Sera Collected from the Veterinary Research Institute Flock.

Hatch	Number of Rous(+) sera[a]				Number of Rous(-) sera[b]				Total number tested	
	Other[c]	VL[d]	NL[e]	OL[f]	Other	VL	NL	OL	Rous(+)	Rous(-)
1948	0	1	1	0	19	4	4	3	2	30
1949	7	0	0	2	23	0	0	1	9	24
1950	1	2	1	0	73	9	2	0	4	84
Total	8	3	2	2	115	13	6	4	15	138

[a]Sera with neutralizing antibodies for the Rous virus.

[b]Sera with no demonstrable antibodies for the Rous virus.

[c]Includes normal birds, cases of bumblefoot, injuries from fighting, ruptured and impacted oviducts, Newcastle disease, airsac infection and chronic sinusitis.

[d]Visceral lymphomatosis.

[e]Neural lymphomatosis.

[f]Ocular lymphomatosis.

Dilutions of sera employed to obtain results in Table 5 varied from 1:4 to 1:50, the latter being used for just a few sera in the earlier part of this study. The amount of Rous virus inoculated varied, from 3 to 140 MIDs, depending on the activity of the different tumor extracts.

C. Flock Hatched from RPL[a] and VRI[b] Stock

Table 6. Results of the Rous Neutralization Tests Carried Out with Sera Collected from RPL and VRI Birds.

Treatment	RPL		VRI	
	Number Rous(+)[c]	Number Rous(-)[d]	Number Rous(+)	Number Rous(-)
Vaccinated[e]	17	21		
Not vaccinated[f]	15	19	30	3
Controls[g]	6	8	15	1
Totals	38	48	45	4

[a]Obtained as trapnested eggs, which were pedigree-hatched, from Line 9 of the Regional Poultry Research Laboratory, East Lansing, Michigan, through Mr. Berley Winton, Director and Dr. N. F. Waters, Geneticist. Only a few cases of lymphomatosis have appeared in the RPL birds hatched at the Veterinary Research Institute. These birds are now about one-year-old.

[b]Pedigree-hatched chicks from inbred White Leghorn families maintained at the Veterinary Research Institute.

[c]Birds with neutralizing antibodies for the Rous virus in their sera.

[d]Birds with no demonstrable neutralizing antibodies for the Rous virus in their sera.

[e]Vaccinated with a Rous virus vaccine, which was treated with formalin, so that 0.2 ml., given subcutaneously, failed to produce tumors in two-week-old chicks. No neutralizing antibodies were demonstrated in sera from three-month-old test birds, bled three and eight weeks after vaccination.

[f]Given no Rous virus.

[g]Isolated unvaccinated birds placed in an individual brooder house at the time the birds in this experiment were vaccinated.

D. Chickens Inoculated with Lymphoid Tumors

Table 7. Results of the Rous Neutralization Tests Carried Out with Sera Collected from Birds Inoculated with Lymphoid Tumors.

Family no.	Number inoculated	NR[a]	Number controls	NR	History and pathology
A-1	4	(-)	1	(-)	VL(c)[b], 5-2-51
B-1	2	(-)	1	(-)	
B-2	2	(-)	2	(-)	
C-1	2	(-)	1	(-)	
D-1	2	(-)	1	(-)	
D-2	5	(-)	3	(-)	Malignant hypernephroma, 12-21-50
E-1	2	(-)	2	(-)	VL(c), 1-22-51
F-1	3	(-)	1	(-)	
F-2	3	(-)	1	(-)	
G-1	4	(-)	1	(-)	
G-2	6	(-)	2	(-)	VL(t)[c], 2-15-51; VL(s)[d], 2-9-51
H-1	4	(-)	2	(-)	
I-1	4	(-)	1	(-)	
I-2	3	(-)	1	(-)	
J-1	3	(-)	2	(-)	
Total	49		21		

[a]Neutralization reaction (12.5 to 62.5 MIDs of virus were used).

[b]Visceral lymphomatosis in an uninoculated control.

[c]Visceral lymphomatosis in an inoculated bird. Tumor suspension from T310, a case of visceral lymphomatosis, was used for inoculation.

[d]Visceral lymphomatosis in an inoculated bird. Tumor suspension from T367, a case of visceral lymphomatosis, was used for inoculation.

E. Experimental Cases of Fowl Lymphomatosis

Table 8. Results of the Rous Neutralization Tests Carried Out with Sera Collected from Experimental Cases of Lymphomatosis in the First and Second Inoculation Series.

Bird no.	Date bled	NR and SD[a]	MIDe[b]	Tumor index	ND/ml[c]	IP[d]	History and pathology
Pooled[e]	9- 7-50	+ 1:100	28	0.0	28,000		
655*						128	VL[f], 10-2-50
654*	10-10-50	+ 1:20	130	0.4	26,000	136	VL, 10-10-50
653*	10-10-50	+ 1:100	130	0.0	130,000		Normal
	12- 6-50	+ 1:100	3	1.2	3,000	193	VL, 12-7-50
652*	10-10-50	+ 1:100	130	0.0	130,000		Normal
	12-12-50	- 1:4	12	4.0			Frozen feet
651*	10-10-50	+ 1:100	130	0.0	130,000		Normal
	4-12-51	- 1:2	12	4.0			"
Control[g]							

[a] Neutralization reaction and serum dilution.

[b] Minimal infective doses.

[c] Neutralizing doses per ml.

[d] Incubation period expressed in days.

[e] Composite serum specimen from the five birds listed in this table.

[f] Visceral lymphomatosis.

[g] Uninoculated controls were raised in a separate building. Losses due to lymphomatosis were less than 4 per cent.

* Birds in the first inoculation series.

Table 8. (Continued).

Bird no.	Date bled	NR and SD	MIDs	Tumor index	ND/ml.	IP	History and pathology
H16**	2-14-51	+ 1:4	12.5	0.0	500	163	VL, 2-23-51
H17**	2-14-51	- 1:4	34.5	4.0			Normal
H18**	2-12-51	- 1:4	111.0	4.0		152	VL, 2-12-51
H19**	1-10-51	- 1:4	62.5	4.0			Lame, not NL[h]
H20**	12-15-50	+ 1:4	12.5	0.2	500		
	12-29-50	- 1:4	6.0	4.0		79	VL, 12-29-50
H21	12-29-50	- 1:4	6.0	4.0			Control[i]
	2-14-51	- 1:4	12.5	4.0		159	VL, 2-19-51
H22	12-29-50	+ 1:4	12.5	0.0	500		Control
H23	2-14-51	- 1:4	12.5	4.0			Control
H24							Lost
H25	3- 1-51	- 1:4	38.6	4.0		188	VL, 4-18-51
							Control

[h]Neural lymphomatosis.

[i]Birds receiving no tumor material but raised with the inoculated birds.

**Birds in the second inoculation series.

Table 9. Results of the Rous Neutralization Tests Carried Out with Sera Collected from Experimental Cases of Lymphomatosis in the Third Inoculation Series.

Bird no.	Date bled	NR and SD[a]	MIDs[b]	Tumor index	ND/ml.[c]	IP[d]	History and pathology
Q53*	2-14-51	? 1:4	38	2.6			Liver enlarged
	3- 6-51	? 1:4	36	2.8		116	VL[e], 4-25-51
Q54*	2-14-51	- 1:4	38	3.6			
	3- 6-51	- 1:4	36	3.6		121	VL, 4-30-51
Q55*	2-14-51	- 1:4	38	4.0			Liver enlarged
	3- 6-51	- 1:4	36	3.6		90	VL, 3-29-51
Q56*	2-14-51	- 1:4	38	3.0			Liver enlarged
	3- 6-51	- 1:4	36	3.4		121	VL, 4-30-51
Q58*	2-14-51	? 1:4	38	2.2			
	3- 6-51	+ 1:4	36	0.6	1440		Died 4-8-51[f]
Q59*	2-14-51	- 1:4	38	3.4		111	VL, 4-20-51
	3- 6-51	? 1:4	36	2.6			
Q60	2-14-51	- 1:4	38	3.4			Uninoculated[g]
	3- 6-51	- 1:4	36	3.6			
Q61	2-14-51	- 1:4	38	4.0			Uninoculated
	3- 6-51	? 2.6	36	2.6			

[a]Neutralization reaction and serum dilution.

[b]Minimal infective doses.

[c]Neutralizing doses per ml.

[d]Incubation period expressed in days.

[e]Visceral lymphomatosis.

[f]Liver appeared enlarged 2-19-51 and 3-2-51, but appeared normal in size at the time of postmortem 4-8-51. Cause of death unknown.

[g]No lymphomatosis has appeared in the controls raised in a separate pen.

*Inoculated birds.

Table 9. (Continued).

Bird no.	Date bled	NR and SD	MIDs	Tumor index	ND/ml.	IP	History and pathology
Q62	3- 6-51	+ 1:4	36	2.0	1440		Uninoculated
Q63	2-14-51	- 1:4	38	4.0			Uninoculated
	3- 6-51	- 1:4	36	4.0			
Q64	2-14-51	- 1:4	38	3.6			Uninoculated
	3- 6-51	- 1:4	36	4.0		142	VL, 5-21-51

F. Pathology

Figures 1, 2, 3 and 4 represent the different types of Rous tumors and the respective quantitative evaluations of neoplastic development employed in determining the tumor indices.

Figures 5 and 6 of the myxosarcoma, T363, exhibit the typical spindle-shaped neoplastic cells, with prominent nuclei, in areas characterized by a large amount of intercellular space. There are also areas of more closely packed cells, without intercellular space, in which the nuclei are more spherical. Similar less differentiated cells were also observed in liver sections of this bird. Grossly, the myxosarcoma involved most all the muscles and subcutaneous tissues of the tibia. This tumor was dull grey, firm and contained irregular neoplastic masses, which, on section exhibited a large amount of a mucinous blood-tinged exudate.

The diagnosis of fowl lymphomatosis was based on gross and microscopic tissue changes. Macroscopic evidence of neoplastic changes were usually present in all cases of spontaneous lymphomatosis, while all experimental cases exhibited marked tumor development.

Figure 7 represents a typical case of experimental visceral lymphomatosis produced through the inoculation of a cellular suspension from the myxosarcoma T363.

Figures 8, 9 and 10 are photomicrographs of neoplastic tissues taken from a bird in each of the first, second and third inoculation series respectively. Numerous neoplastic cells, apparently undifferentiated lymphocytes, were usually present in the liver, spleen, kidneys

and bone marrow of each bird. Figure 10, from one of the birds in the third series, indicates a less rapid degree of tumor development. Foci of tumor cells were observed in sections of the liver and spleen but not in the kidneys and heart. All the other birds in the third series exhibited a more acute, diffuse type of neoplastic development.

Figure 1. A 4+ Rous tumor development in the chicken, produced by the subcutaneous inoculation of 0.2 ml. of Rous virus, after an incubation period of thirty days.

Figure 2. A 3+ Rous tumor development in the chicken, produced by the subcutaneous inoculation of 0.2 ml. of Rous virus, after an incubation period of thirty days.

Figure 3. A 2+ Rous tumor development in the chicken, produced by the subcutaneous inoculation of 0.2 ml. of Rous virus, after an incubation period of thirty days.

Figure 4. A 1+ Rous tumor development in the chicken, produced by the subcutaneous inoculation of 0.2 ml. of Rous virus, after an incubation period of thirty days.

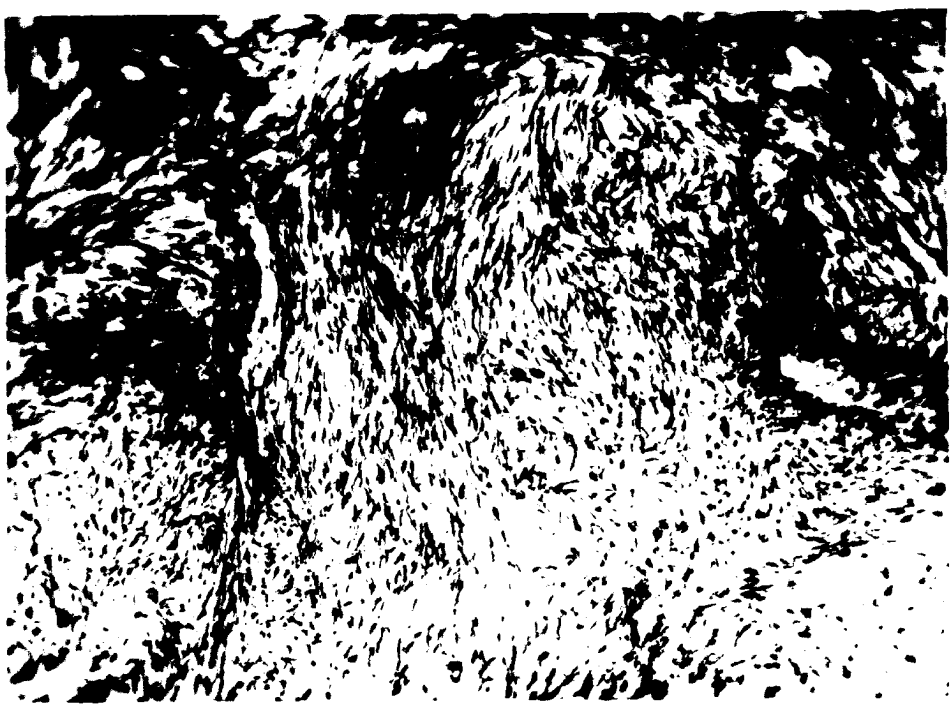

Figure 5. Myxosarcoma T363. This tumor served as the source of a cellular inoculum which produced visceral lymphomatosis in the chicken. X125.

Figure 6. Myxosarcoma T363. This tumor served as the source of a cellular inoculum which produced visceral lymphomatosis in the chicken. X500.

Figure 7. Experimental visceral lymphomatosis, T718, produced by the inoculation of a tumor extract from a bird in the second inoculation series (T633).

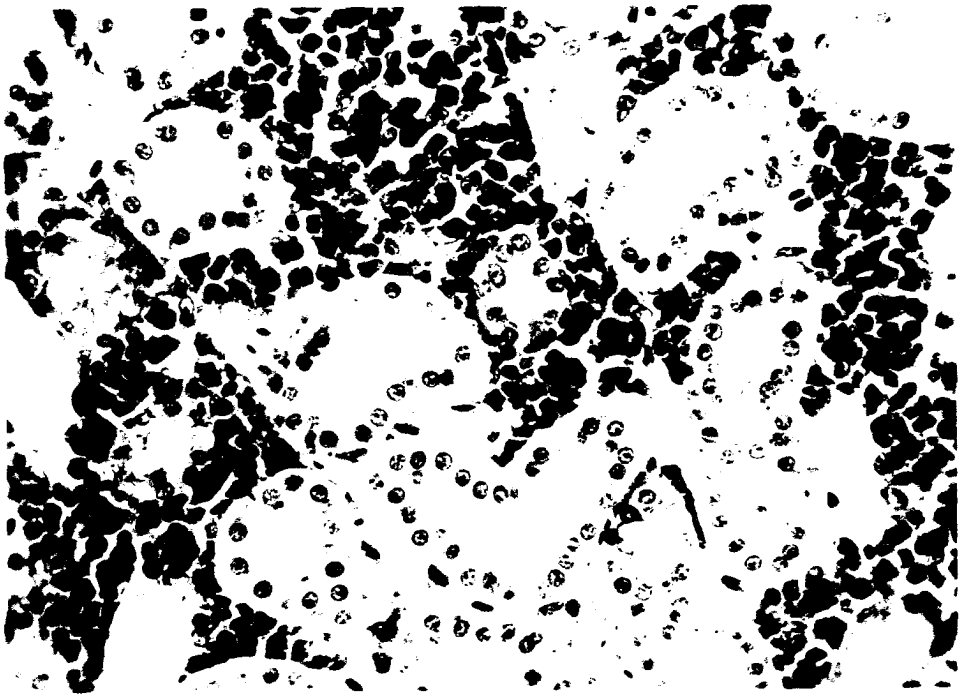

Figure 8. Experimental visceral lymphomatosis T473. Neoplastic renal tissue from Chicken 654 in the first inoculation series. This bird was inoculated with a cellular suspension of the myxosarcoma (T363). X500.

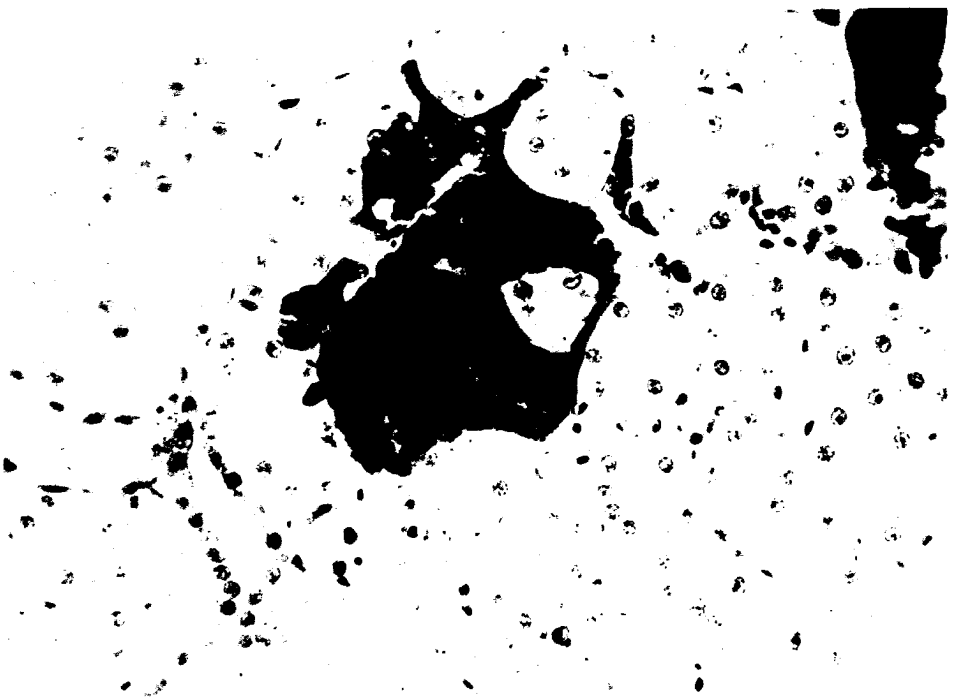

Figure 9. Experimental visceral lymphomatosis T633. Neoplastic renal tissue from Chicken H20 in the second inoculation series. A cellular suspension from T473 (Figure 8), served as an inoculum for this series. X500.

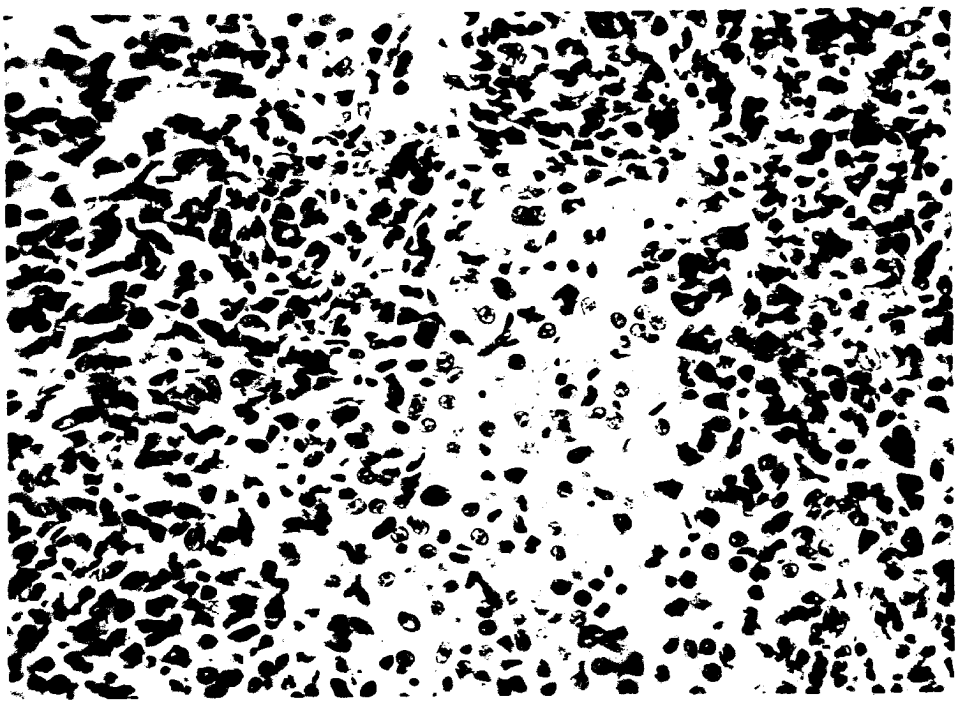

Figure 10. Experimental visceral lymphomatosis T680. Neoplastic hepatic tissue from Chicken Q55 in the third inoculation series. A cellular suspension from T633 (Figure 9), served as an inoculum for this series. X500.

V. DISCUSSION

The Rous neutralization test (RNT) appears to give consistent results as indicated by comparison of several sera from the same bird (Table 1). The wide range in the number of minimal infective doses (MIDs) inoculated during this study (3-140) must be considered when the neutralization test is interpreted. A fresh tumor extract was prepared each week, since inactivation of the virus occurred rapidly and consistent activity had not been maintained by dry-freezing or by freezing.

A tumor index (TI) of over 2.0 is usually not considered as evidence of neutralization; however an index of 2.4-2.6 in birds whose titers are decreasing or increasing may suggest at least partial neutralization. A more satisfactory answer is generally available after comparison of several tumor indices obtained over a period of several months.

Hen 60, Table 1, when bled 10-28-50, showed some evidence of neutralizing antibodies against 35 MIDs with a TI of 2.4. The possibility of a more clear-cut reaction against less than 35 MIDs of virus should be considered. It is evident that reference must be made to the amount of Rous virus employed when a bird's serum is considered lacking in neutralizing properties.

Birds Q44, Q45 and Q46 (Table 4), were kept in the same brooder-pen with Q42 for several months. On the basis of the RNT run 10-1-50 and 10-28-50, it appears that some agent may have been transmitted by Q42; however the lack of neutralizing data for 25 MIDs of virus on 10-1-50 sera interfers with a satisfactory interpretation. It is interesting to

note that Q46 died of visceral lymphomatosis about a week after negative sera was collected.

Chickens, apparently normal, may exhibit high levels of neutralizing antibodies against the Rous virus (Tables 1, 4, 5, 6 and 8). This has been shown by Andrewes (1933) and (1939), McMaster et al. (1934), Amies (1937), and Duran-Reynals (1940).

The incidence of neutralizing titers in Flocks S (Table 1) and 454, 494 and 526 (Table 2), where severe losses due to lymphomatosis occurred, is many times greater than that in Flocks RG (Table 3) and VRI (Table 5), where such losses were light.

Some flocks such as S (Table 1), 494 (Table 2), VRI (Table 5) and the experimental cases in Table 8 exhibit birds from the same flock dying of visceral lymphomatosis with high neutralizing titers, as well as birds with very low or no neutralizing titers. One might expect a lack of neutralizing properties for a disease which is destroying the host. Such neutralizing antibodies might be formed against a substance produced by the agent in attacking the host's cells, rather than against the agent itself. However since an incubation period is required for the neutralizing action upon the virus, the antibodies would appear to be antiviral, Duran-Reynals (1940).

The matter of individual variation in susceptibility between birds of a flock must be considered. This is shown in Flock S (Table 1), where less than 40 per cent of the birds failed to exhibit RNA in the tests carried out. Are these Rous negative birds refractory or did they once develop neutralizing antibodies in their sera? Some of the birds referred to in Table 6 exhibited RNA at twelve weeks of age, later becoming

negative when nine months old. The sera from Q42 (Table 4) has lost much of its neutralizing properties. Thus it would appear important to test sera when the birds are six to eight months of age, if attempts are made to reduce the incidence of tumors on the basis of the RNT.

If the viral agent must be present in the bird's tissues to maintain an antibody level, then recovered birds with no neutralizing antibodies might not be considered as carriers. The RNA present may be expected to neutralize or mask the agent under certain conditions, as observed by Claude (1937), Carr (1942) and Duran-Reynals (1950).

The data in Tables 8 and 9 suggest that some of the control birds in the second and third series, kept in the same pen with inoculated birds, may have developed RNA as the result of some transmitted agent.

Duran-Reynals (1950) referred to the development of RNA in birds raised in the same room with birds bearing tumors of different types.

Data in Table 7 indicate that visceral lymphomatosis may occur in a flock where no RNA have been demonstrated against 12.5 MIDs of Rous virus. These families are certainly susceptible since their sibs (VRI birds in Table 6) demonstrated over 90 per cent infectivity as indicated by the presence of RNA. Similarly, the RG Flock (Table 3) failed to exhibit RNA, even though a number of lymphomatosis cases were observed. The possibility of more than one type of visceral lymphomatosis should be considered. Feldman and Olson (1948) suggested that several entities may be concerned as not all of the lymphoid tumors are transplantable. Certainly the tumor suspensions employed for inoculation of the birds in Table 7 have not increased the incidence of tumors. Possibly these lymphoid tumors should be classed as lymphocytomas. Such factors as

the susceptibility of the inoculated stock and the virulence of the agent must also be considered. Data from Tables 8 and 9 certainly indicate that birds of the same population were susceptible to the agent employed. Evidently some of the lymphoid tumors must be of very low virulence or not transmissible under the experimental conditions of this study.

A review of the data presented in Table 8 indicates that the tumor suspension from a myxosarcoma was capable of producing RNA in at least 80 per cent of the birds inoculated. The incidence of visceral lymphomatosis was 60 per cent in this series, while the spontaneous incidence of the lymphomatosis-complex in the control flock, kept in a separate building, was less than 4 per cent. The agent producing lymphomatosis appears to have been present in the tumor suspension prepared from the myxosarcoma. The bird bearing the myxosarcoma originated from a flock where early significant losses due to lymphomatosis were occurring.

A 60 per cent loss due to visceral lymphomatosis also occurred in the second series (Table 8), while the controls in the same pen experienced a 40 per cent loss. Rous neutralizing antibodies were irregularly present in both the inoculated and control birds. Some agent appears to have been transmitted to the control birds.

The third series (Table 9) lost over 80 per cent of the birds due to visceral lymphomatosis, while the average incubation period of 112 days suggests an increased virulence of this agent. The sporadic occurrence of RNA is difficult to explain in view of the consistent response in the first series. Possibly the agent capable of producing RNA is being

diluted and lost through serial transmission of the lymphoid tumor.

Bird Q58 (Table 9) offers an interesting case in view of the apparently enlarged liver observed some time after inoculation, but not present at the time of postmortem. Since no laparotomy was performed, the absolute presence of the liver enlargment was not established. Davis and Doyle (1947) have referred to recovered cases of visceral lymphomatosis based on liver biopsy. The TI and history of Q58 might suggest that this infection must be present some time before RNA appear and possibly that regression or recovery may favor their appearance.

The role of the egg in transmitting lymphomatosis has been established by Cottral (1949). The data in Table 6 indicate that some agent, capable of stimulating RNA, was transferred from the Lansing eggs to the Lansing chicks. It is doubtful that RNA observed three to four months after hatching were transferred through the yolk, since Andrewes (1939) reported their presence for only a few weeks after hatching. The presence of such antibodies in the VRI population are due to the transmission of this agent either at the time of hatching or during contact when the birds were raised together. The sibs of these VRI birds and the observations in Table 7 indicate that RNA did not occur spontaneously in these families. The isolated controls, listed in Table 6, eliminate the chance that the Rous virus vaccine was responsible for the presence of these antibodies.

Table 7 also suggests that tumor suspensions from some birds with visceral lymphomatosis and no history of demonstrable RNA, fail to stimulate the formation of such antibodies in chickens. While Table 8 suggests that some agent in certain cases of visceral lymphomatosis,

where RNA have been demonstrated, is capable of stimulating the formation of RNA.

The low incidence of lymphomatosis in the RPL birds of Table 6 is difficult to explain since their dams experienced a 41 per cent incidence of this disease in two years, Winton (1949). However, the RPL birds have been under observation for just one year at the Veterinary Research Institute. Possibly the 20 per cent loss due to coccidiosis and Newcastle disease may have selected those birds most susceptible to lymphomatosis. It is doubtful that the attenuated Rous virus would affect the incidence of this disease. Carr (1942) reported that birds developing Rous tumors, which regress, may die of this tumor as much as a year later, so it questionable whether a satisfactory immunity ever develops. On the basis of Hutt and Cole's report (1951), the incidence of lymphomatosis should be greatly reduced since these chicks were raised in isolation. However, after the age of five weeks, the birds were cared for by caretakers of the adult flock and raised within 60 ft. of the grown birds. Lymphomatosis was present in the adult flock.

Rous neutralizing antibodies are most often associated with the visceral type of lymphomatosis (Tables 1 and 2). Occasional birds with the ocular and neural forms may also exhibit these antibodies (Tables 2 and 5). However, the osteopetrotic cases, which came from flocks with a very low incidence of the lymphomatosis-complex, failed to demonstrate RNA. This high incidence of RNA in Flocks S, 454, 494 and 526, where severe losses due to lymphomatosis have occurred, may suggest an antigenic relationship to the Rous sarcoma. Many of the connective tissue tumor agents appear to be antigenically related, Andrewes (1933),

however no report of such a relationship to the lymphoid tumor agent has been shown. Cottral and Winton (1948) and Winton (1950) discussed the possibility of such a relationship. Some of the other tentative explanations should include: (1) concurrent infections of the lymphoid tumor agent group and the connective tissue tumor agent group, obtained through the egg, from infected eggs at the time of hatching or through contact with infected older birds; (2) a synergistic relationship between lymphoid tumor agents and connective tissue tumor agents, which is expressed by a high incidence of visceral lymphomatosis; and (3) the neoplastic potentiality of the connective tissue tumor agent group to attack lymphoblastic cells or precursors of the lymphocytic series.

Many investigators, McIntosh (1933), Andrewes (1936), Carr (1942), Greenwood and Peacock (1945) and Burmester (1947) have all emphasized the possibility of several tumor agents being present in an avian population. The review of Murphy and Claude (1935), as well as the reports of Stubbs and Furth (1935), Furth (1936a), (1936b), Duran-Reynals (1946), and Burmester et al. (1946) further emphasize the widespread distribution of tumor producing viruses in chickens. Consequently the concept of concurrent tumor virus infections should be quite acceptable. Possibly a greater incidence of connective tissue tumors should be expected in these populations characterized by a high incidence of RNA.

A number of synergistic relationships exist in certain bacterial and viral diseases, such as canine distemper, swine influenza and human influenza. Certainly the high incidence of RNA in flocks suffering heavy losses from visceral lymphomatosis would not preclude a

synergistic relationship of connective tissue and lymphoid tumor agents. The high incidence of visceral lymphomatosis produced in the experimental birds, referred to in Tables 8 and 9, through the inoculation of a connective tissue tumor extract, may be the result of such a synergistic relationship.

The concept of pleomorphic tumor viruses has been advanced by McIntosh (1933), Oberling and Guérin (1933), Rothe Meyer and Engelbreth-Holm (1933) and Stubbs and Furth (1935). The ability of a connective tissue tumor agent to produce a lymphoid tumor should be considered from the data in Tables 8 and 9. However, no connective tissue tumors have been observed in any of the sub-inoculations. Such tumors may not be necessary to indicate the presence of a virus, since Carr (1942) found that Rous virus may exist latently for almost a year in experimental birds.

Further investigations are indicated in order to obtain a better understanding of the lymphomatosis-Rous sarcoma relationship. Some of these are: (1) the preparation of a cell-free extract of the Rous sarcoma which would maintain more consistent activity, thus aiding in the evaluation of neutralizing properties present in the sera; (2) collect more data on the incidence of RNA in flocks with severe losses due to fowl lymphomatosis, as well as, those experiencing minor losses; (3) attempt to transmit lymphoid tumor extracts, from different well-known flocks, to suitable numbers of properly isolated and controlled birds; (4) correlate the incidence of RNA, obtained from sera collected at regular intervals, with the history of each bird; (5) utilize the liver biopsy technique, reported by Davis and Doyle (1947), in collecting

more information concerning the recovery of a bird from lymphomatosis; (6) compare the incidence of fowl lymphomatosis in birds separated on the basis of the RNT, as well as, the incidence of this disease in their progeny; and (7) the study of more connective tissue tumors in respect to their ability to produce visceral lymphomatosis and to stimulate the formation of RNA.

VI. SUMMARY AND CONCLUSIONS

1. Over 60 per cent of the sera from field flocks with 30 to 50 per cent losses due to fowl lymphomatosis contain Rous neutralizing antibodies (RNA).

2. Where losses due to fowl lymphomatosis are less than 5 per cent the incidence of RNA is less than 10 per cent, while no antibodies have been demonstrated in sera from some of these populations.

3. Rous neutralizing antibodies are present in over 40 per cent of the sera of the birds hatched from a population which experienced a 41 per cent loss due to fowl lymphomatosis in two years.

4. Cases of fowl lymphomatosis (lymphocytomas?) were observed in a population where no RNA were demonstrated.

5. The incidence of RNA appears greatest in birds with the visceral type of lymphomatosis. These antibodies have also been observed in sera from cases of ocular and neural lymphomatosis, but not from cases of the osteopetrotic type.

6. A cell-containing suspension of a myxosarcoma has been shown to produce visceral lymphomatosis in three serial sub-inoculations. Rous neutralizing antibodies were consistently present in the first inoculation series and occasionally in the second and third series.

7. A method, based largely on the technique of Duran-Reynals (1940) and (1949), for determining the presence and evaluating the quantity of RNA is outlined.

8. The transmission of the agent causing visceral lymphomatosis through contact is substantiated.

9. The transmission of an agent through the egg, capable of stimulating RNA, is shown.

10. The transmission of an agent through contact, capable of stimulating RNA, is shown.

11. Some of the experimental evidence obtained in this study supports the concept of non-transmissible lymphoid tumors.

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